ERBB2 IN MOUSE MODELS OF MAMMARY GLAND DEVELOPMENT AND TUMORIGENESIS

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Amplification and the consequent overexpression of neu (c-erbB-2, Her-2) has been observed in 20-30% of human breast cancers. Consistent with these clinical observations, expression of the neu oncogene under the transcriptional control of the mouse mammary tumor virus (MMTV) long terminal repeat results in the rapid induction of multifocal mammary tumors in transgenic mice. Although these mouse models have illustrated the role of Neu in the induction of mammary tumors, Neu expression in these models is driven by a strong viral promoter of questionable relevance to the human disease. The purpose of this study was to examine the role of activated Neu when expressed under the endogenous promoter. Additionally, since the role of Neu in mammary gland development is poorly understood, we have also examined mammary development in the absence of Neu.

To ascertain whether expression of activated Neu under the control of the endogenous promoter in the mammary gland could induce mammary tumors we have generated mice that conditionally express activated Neu under the transcriptional control of the intact endogenous Neu promoter. In a similar fashion, we also generated mice that will conditionally inactivate Neu using MMTV-Cre transgenics to drive excision of the loxP flanked Neu cDNA.

Interbreeding the MMTV-Cre transgenics with mice carrying an inducible activated Neu allele resulted in mammary gland specific activation of the recombinant allele. Conditional activation of Neu in the mammary gland resulted in accelerated lobulo-alveolar development and altered branching. Formation of focal mammary tumors occurred after a long latency period. Interestingly, all mammary tumors bear amplified copies (2-22 copies) of the activated neu allele relative to the wild-type allele and express highly elevated levels of neu transcript and protein. In addition to the chromosomal amplification, we have also observed double minute chromosomes containing Neu. Conditional inactivation of Neu resulted in a defect in ductal outgrowth in the early stages of development. These results have provided a more relevant mouse model of mammary tumorigenesis for breast cancer research and have illustrated the role of Neu in mammary gland development.

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DETERMINING IN VIVO THE ROLE OF EPIMORPHIN IN MAMMARY GLAND DEVELOPMENT

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Epimorphin/syntaxin-2 (EPM) is an extracellular, membrane associated, mesenchymal /myoepithelial morphogen that induces mammary epithelial cells (MECs) to differentiate into branching or lobuloalveolar structures depending on the orientation of presentation. When EPM is presented to MEC clusters in a polar fashion on the basolateral cell surface, the cells exhibit branching morphogenesis. In contrast, when EPM is clustered with the MECs so as to be presented in an apolar fashion, that is accessible by all sides of the cell, the cells develop into acinar like structures with central lumens. Here we have utilized wildtype mice and a transgenic mouse model, previously generated by Dr. Bissell's laboratory, that expresses EPM in an apolar fashion on the cell surface of MECs under the control of the whey acidic protein (WAP) promoter, to investigate the factors that control EPM induction *in vivo,* and the effector molecules that affect the localization and spatial orientation of EPM. I have set out to define how EPM induces functional morphogenesis of the mammary gland in *vivo*. By whole mount analysis and hematoxylin and eosin stained mammary gland sections, I have determined that WAP-EPM mice have a strong mammary gland phenotype at midgestation and during metestrus in nulliparous mice marked by precocious lobuloalveolar development, thick ducts and extensive lateral branching. My results support the possibility that EPM expression increases the branching response in the nulliparous mammary gland and the lobuloalveolar phenotype during pregnancy and lactation. To determine the role of EPM in normal mammary gland development, I used an outbred strain of wildtype mice, CD-1, as a source of tissue at distinct mammary gland stages for analysis of expression and localization of EPM, using quantitative real-time PCR and quantitative western blotting to determine gene expression and protein expression, and using immunofluorescence and in situ hybridization to determine the localization of EPM. Our results suggest that EPM plays a role in branching and lobuloalveolar development in the nulliparous and midpregnant mammary gland and may play a regulatory function in lactation.

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GLUTATHIONE PEROXIDASES IN THE NORMAL MOUSE MAMMARY GLAND

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Ovarian hormones are known to influence susceptibility to mammary carcinogenesis. Women without ovaries have a low risk of breast cancer, while prolonged ovarian activity during life (early menarche or late menopause) is associated with increased risk. Proliferative activity induced by ovarian hormones, increasing the circumstances in which genetic alterations could occur, is considered to be an important determinant of mammary carcinogenesis. However, there is increasing evidence that oxidative stress, linked to estrogen metabolism and hormonal status, may lead to DNA damage and contribute more directly to mammary carcinogenesis. Antioxidant defense systems utilizing low molecular-mass scavengers such as glutathione and enzymes including glutathione peroxidase (Gpx) protect against harmful reactive oxy-intermediates and there is evidence linking Gpx genes with ovarian hormone action. Gpx activity is elevated in erythrocytes during the menstrual cycle and ovarian hormones regulate the expression of Gpx1, in the corpus luteum and the endometrium. Therefore, hormonal regulation of Gpxs may represent a general mechanism to limit the oxidative damage caused by ovarian hormones in a range of target tissues.

We have shown, by reverse transcriptase PCR, that at least four members of the Gpx family, Gpx1, 2, 3 and 4, are expressed in the normal mammary gland of BALB/c mice. The expression of one Gpx in particular, Gpx3, was found to be up-regulated in response to ovarian hormones. Analysis by cDNA array showed that Gpx3 was highly expressed in the normal mammary gland and induced 3.7-fold by estrogen (1 ug) and progesterone (1 mg), at 6 hours. This was subsequently confirmed by real-time, quantitative PCR and Gpx3 was also found to be up-regulated by estrogen alone, in a dose-dependent manner. We propose that Gpx induction is one mechanism to self-limit hormonally-induced oxidative damage. If this is the case, then a decrease in Gpx activity will lead to an increased susceptibility to oxidative DNA damage and eventually to abnormalities in the mammary gland.

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PCAF AND GCN5 IN MOUSE MAMMARY GLAND DEVELOPMENT

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PCAF and GCN5 are two highly related proteins which possess histone acetyltransferase and transcriptional regulatory activities. PCAF interacts with many transcriptional regulatory molecules known to be important in normal mammary gland development and in breast cancer. These include the estrogen and progesterone receptors, p53 and amplified in breast cancer-1. PCAF also interacts with p300, CBP and steroid receptor coactivator-1. Analysis of mice with homozygous deletions of PCAF and GCN5 revealed distinct functions for the two proteins. PCAF-deficient mice are viable and fertile. GCN5-deficient embryos die in utero by day 10.5 post coitus.

Because of the known interactions between PCAF and the steroid hormone receptors and the high degree of homology between PCAF and GCN5, we hypothesize that PCAF and GCN5 are important to mammary gland development and carcinogenesis. We are testing this by analyzing PCAF and GCN5 expression during mouse mammary gland development and carcinogenesis and by analyzing mammary gland development in the absence of PCAF or GCN5.

During all stages of mouse mammary gland development, mRNA for both GCN5 and PCAF can be detected. PCAF-null mice show delayed ductal development of the virgin mammary gland during puberty as compared with wild type siblings. Preliminary data indicates that mature PCAF-null mammary epithelium differs from wild type epithelium in its ability to proliferate in response to an acute administration of exogenous estrogen and progesterone. We are testing the hypothesis that estrogen action is decreased in the absence of PCAF resulting in delayed development during puberty and decreased responsiveness to exogenously administered steroids. PCAF mRNA is elevated during involution. The PCAF null mice are currently being examined for defects in involution, lactation and lobuloalveolar development during pregnancy. GCN5 heterozygous mice do not display any mammary gland defects. A mammary specific deletion of GCN5 using Cre-Lox technology is in progress.

These investigations should elucidate how the actions of PCAF and GCN5 influence normal mammary gland development and how alterations in the levels or activities of these proteins may contribute to the process of mammary carcinogenesis.

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A TRANSGENIC ANIMAL MODEL TO STUDY THE ROLE OF MAMMARY MYOEPITHELIAL CELLS IN GLANDULAR DEVELOPMENT AND LACTATION

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Studies indicate that early pregnancy is protective against mammary cancer in rodents and humans. The precise mechanisms underlying this protection are unclear, but increased structural and functional differentiation of the glands is involved. The mammary myoepithelium is composed of specialized cells that surround ducts and acini and express proteins characteristic of both smooth muscle and epithelium. Myoepithelial cells lay down basement membrane and contribute to the structural integrity of the gland. Studies also indicate that the myoepithelium may impact cancer progression by providing a physical and biochemical barrier to invasion. To study the role of myoepithelium in regulating glandular development, functional differentiation and cancer susceptibility, we developed transgenic mice in which E2F-1 overexpression is controlled by the K5 promoter and thereby directed to the myoepithelial cells of the mammary gland. In order to produce transgenics on a defined background (congenic) and to facilitate carcinogenesis experiments, animals were backcrossed onto C57Bl, Balb/c and SSIN inbred strains. Some background lines demonstrated poor fecundity and/or failure to thrive and were discontinued. Transgenic females were fertile but unable to nurse pups.

To evaluate if K5 directed transgenes would be expressed from birth, we performed K5 and K8 double fluorescent immunostaining of mammary buds from newborn and 2.5 day old pups. In both cases, uniquely staining K5 positive myoepithelial cells and K8 positive glandular epithelial cells were present in the expected spatial orientation in the early ductal structures.

Glands from post-pubertal, virgin E2F-1 transgenics of all three lines had profoundly reduced ductal elongation, branching and alveolar bud formation compared to age-matched wild type littermates. Mammary glands from sexually mature, virgin E2F-1 transgenics had significantly increased levels of proliferation compared to wild types as measured by BrdU incorporation. Virgin glands from Balb/c and C57Bl also had increased levels of apoptosis, determined by TUNEL analysis. This lack of development was only partially overcome in pregnant animals, where a normal lactational phenotype with full alveolar formation was not achieved. Mammary phenotypes varied between congenic lines, with pregnant Balb/c mice achieving the greatest level of alveolar development. The mammary glands from pregnant SSIN congenics displayed the greatest level of structural disruption and had significantly increased levels of apoptosis and proliferation compared to pregnant wild type littermates.

These data suggest that overexpression of E2F-1 stimulated increased cellular turnover that profoundly reduced or delayed mammary development. These results indicate that myoepithelial cells play a pivotal role in directing structural and functional differentiation of the mammary gland, and may, therefore, be an important mediator of susceptibility to

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NEU AND ANDROGEN RECEPTOR REGULATE CELLULAR FATE IN THE MAMMARY GLAND

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The non-mutated HER-2/neu proto-oncogene is amplified and overexpressed in approximately 25% of human breast cancers, yet its precise role in breast carcinogenesis is poorly understood. This is due partly to a lack of animal models that overexpress the non-mutated neu proto-oncogene. We therefore created transgenic rats to target the neu proto-oncogene to the mammary gland using the mouse mammary tumor virus promoter. High levels of neu protein were detected equally in the mammary epithelium of both transgenic males and females. However, mammary cancer was induced only in the male transgenic rats, occurring with a 100% incidence. Unlike in previous transgenic mouse models, mammary carcinomas were not associated with mutations in the neu proto-oncogene. These cancers displayed epithelial expression of the androgen receptor (AR). Orchidectomy of transgenic males while young adults was sufficient to completely inhibit mammary carcinogenesis. Chronic androgen treatment restored mammary carcinomas to 100% of orchidectomized transgenic males. Most significantly, 75-80% of ovariectomized transgenic females developed mammary cancer if similarly treated with androgens. Established male mammary carcinomas initially regressed after orchidectomy, but eventually reemerged as AR-negative tumors. Apart from their effects on carcinogenesis, androgens and neu were found to be very important in regulating the differentiation of the adult rat mammary epithelium. Neu overexpression in the transgenic rat substituted for androgens in maintenance of the male mammary morphology following orchidectomy. Furthermore, neu overexpression induced the mammary epithelium of the transgenic ovariectomized female to adopt a male morphology. When neu levels were not elevated, chronic androgen treatment induced partial transdifferentiation of the mammary epithelium to ventral prostate in 64-78% of the non-transgenic male and female rats. The overexpression of neu in the transgenic rats caused a significant reduction in the incidence of transdifferentiation to just 15-20%. This work revealed that interactions occur between the neu and AR signaling pathways, which can have profound effects on cellular development and differentiation within the adult mammary gland.

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DISRUPTION OF STEROID AND PROLACTIN RECEPTOR PATTERNING IN THE MAMMARY GLAND CORRELATES WITH A BLOCK IN LOBULOALVEOLAR DEVELOPMENT

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The study of genetically engineered mice has helped elucidate the function of genes that are critical regulators of mammary gland development. Previously, targeted deletion of the bZIP transcription factor, C/EBPß, has been shown to result in aberrant ductal morphogenesis and decreased lobouloalveolar development, accompanied by an altered pattern of progesterone receptor (PR) expression. Similar changes in the level and pattern of prolactin receptor (PrlR) expression were also observed while screening for additional changes in gene expression in C/EBPß-null mice. Alterations in the patterns of PR expression were also detected in PrlR-null mice, as well as in mammary epithelial cell transplants from both PrlR-null and Stat5a/b-deficient mice, with concomitant defects in hormone-induced proliferation. Progesterone treatment induced PrlR expression in wildtype, but not PR-null mice. Downregulation of PR and activation of Stat5 tyrosine phosphorylation was seen following estrogen and progesterone (E+P) treatment in both C/EBPß-null and wildtype mice, indicating that these signaling pathways were functional despite the failure of steroid hormones to induce proliferation. Another differentially regulated gene, IGFBP-5, displayed both an altered pattern and decreased expression in C/EBPß-null mice. In addition, SPRR2A expression, a marker of epidermal differentiation, was detected in the mammary epithelium of the C/EBPß-null mice. Together these data suggest that C/EBPß regulates mammary epithelial cell fate and that the correct spatial pattern of PR and PrlR expression is a critical determinant of hormone-regulated cell proliferation.

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WHN GENE MUTATION RESULTS IN IMPAIRED MAMMARY GLAND DEVELOPMENT

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Loss-of-function mutations in the Whn transcription factor result in the nude phenotype, which is best known for its characteristic athymia, lack of visible hair, and defects in the epidermis. In addition to these abnormalities, nude female mice, although fertile, fail to lactate sufficiently to nourish their pups. This study was undertaken to investigate the function of Whn in mammary gland development.

The role of Whn in mammary gland morphogenesis was assessed using the following experimental approaches: (1) analysis of nude vs. wild-type mammary gland development, and (2) determination of the temporal and spatial expression of Whn in mammary glands from wild-type virgin, pregnant and postpartum mice.

Comparison of mammary glands from either wild-type or nude mice showed that the terminal end buds form later in nude juveniles than in normal littermates, and the degree of ductal branching is significantly reduced in mature nude virgins. During pregnancy, the nude mammary gland matures to only one-third the size of a normal gland. One day postpartum, the nude gland possesses small lobulo-alveoli that remain full of secretions despite the presence of pups attempting to suckle. The nude alveolar epithelium also appears thinner, and in some cases, the myoepithelial layer seems to degenerate. These results suggest defects in mammary gland growth and differentiation. These defects appear intrinsic to mammary epithelium, since there is no reduction in the levels of circulating steroid hormones. In normal mammary glands, whn transcript is found at times when the tissue is actively growing and differentiating. On a nude genetic background, the expression of a whn transgene in mammary epithelial cells rescued the lactation defects.

We conclude that the delay in the maturation of glands, and the failure to lactate in the nude mice is due to intrinsic defects of the mammary epithelial cells. This study indicates that Whn function is important in both the proliferation and differentiation of the mammary epithelial cells.

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TRANSCRIPT PROFILING OF PROLACTIN-DEPENDENT MODELS OF MAMMARY DEVELOPMENT REVEALS GENES IMPORTANT FOR LOBULOALVEOLAR DEVELOPMENT

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Analysis of prolactin receptor knockout (PRLR-/-) mammary epithelium has shown that prolactin promotes lobuloalveolar development during pregnancy. We wanted to determine if PRLR's in the stroma played a role in mammary development and to dissect the molecular events following prolactin action on the mammary gland during pregnancy.

To do this mammary recombination experiments were performed whereby mammary fat pads from PRLR+/+ or PRLR-/- mice were recombined with PRLR+/+ or PRLR-/- epithelium and transplanted into Rag1-/- host animals. Transcript profiling using the Affymetrix system was performed on PRLR+/+ or PRLR-/ epithelial transplants at 2, 4 and 6 days of pregnancy. Rag1-/- fat pads cleared of epithelium were also transcript profiled to allow transcripts to be assigned to the epithelial or stromal compartment of the gland. Analysis was performed using Microarray Suite and transcript profiles compared using principal components analysis.

Mammary recombination demonstrated that prolactin acts entirely through the epithelium and not through the stroma to promote lobuloalveolar development during pregnancy. Approximately 50% of the 10 000 transcripts profiled were called present in each mammary gland by Affymetrix. The greatest number of transcripts were present at day 4, a period of high proliferation in the gland. Principal components analysis confirmed global differences between PRLR+/+ and PRLR-/- glands. Epithelial transcripts were called absent in the cleared fat pad and present in PRLR+/+ glands. On average across the days 15% of genes present in the PRLR+/+ epithelium were decreasing in the PRLR-/- epithelium. These genes are important for prolactin dependent lobuloalveolar development, a number of which have previously been shown to be important for mammary gland development. Mice lacking osteoprotegrin ligand, tyrosine phosphatase LAR or amphiregulin all exhibit mammary gland defects at the stage of lobuloalveolar development. Affymetrix calls were confirmed by LightCycler analysis of a number of these genes.

In conclusion we have used a prolactin dependent model of mammary development to discover genes important for lobuloalveolar development during pregnancy which will be analyzed for relevance to breast cancer treatment outcome.

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AGE-RELATED DECLINE IN SERUM AND PINEAL MELATONIN LEVELS IN BUFFALO RATS

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Over the last two decades, considerable evidence has accumulated demonstrating that the pineal gland, via its hormone melatonin, possesses significant oncostatic activity, particularly for breast cancer. Our studies have demonstrated that melatonin is able to significantly suppress the growth of breast cancer cells through the activation of the MT1 melatonin G-protein receptor. Given that melatonin levels diminish significantly by the 5th and 6th decades of life as the incidence of breast cancer increases, we hypothesize that the age related decline in pineal melatonin production leads to an enhancement of breast cancer development and growth in older women. Given that there are no well designed or tested models of aging and breast cancer the purpose of these studies is to define the age-related changes in melatonin, its receptor receptors, and thus, the growth of mammary tumors in young, middle-aged and old female Buffalo rats.

To begin to define the melatonin profile in Buffalo rats, serum melatonin was measured at 0900 and 1600 h (light phase) and 1800, 2200, 2300, 2400, 0100, 0200 and 0400 h (dark phase) of 10 young (8 months of age) and 10 middle-aged female rats (15 months of age). In adult rats the onset of the evening melatonin rise was delayed by approximately 2-3 h. This delay in the onset of the melatonin plateau was also accompanied by a significant ($p < 0.05$) decrease (29%) in the peak value of serum melatonin (mean peak melatonin serum level of 123 pg/ml and 88 pg/ml of serum in young and adult rats, respectively) in middle-aged rats. The level of pineal melatonin also showed a significant diminution ($p < 0.01$) of night-time pineal melatonin levels in young vs. middle aged rats. The night-time melatonin content of the pineal glands of your rats exceeded daytime levels by 13-fold, whereas in the middle-aged rats only a 7-fold increase in nocturnal pineal melatonin was observed.

It has been well documented that melatonin can modulate uterine function in hamsters. Our studies demonstrate that the uteri of female Buffalo rats express quantifiable levels of the MT1 melatonin receptor and that the levels of this receptor were diminished by 41% in adult rats compared to young rats.

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LOCAL REGULATION OF MAMMARY GLAND GROWTH BY OVARIAN AND PITUITARY HORMONE INTERACTIONS

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Mammary gland growth and function during development is tightly regulated by a co-ordinated balance of systemic hormones including the ovarian hormones, estrogen (E) and progesterone (P), and the pituitary hormone, prolactin (PRL). The aim of this research is to elucidate the mechanisms by which these hormones interact to elicit the growth and morphogenesis of the mammary epithelium in nulliparous females as a basis for understanding the onset of neoplastic transformation. As a first step toward understanding the role of PRL, the expression and distribution of PRL receptors (PRLR) in the developing mammary gland was determined. Expression of PRLR mRNA in the mammary gland increased during puberty to maximum levels in mature nulliparous females, then declined during pregnancy. Levels subsequently increased during early lactation and further increased during involution. The existence of stromal PRLR was examined in cleared mammary fat pads at different ages. Whereas the long form of the PRLR was expressed at a constant level, three short forms were most highly expressed in the stroma of neonates, with expression subsequently declining at different rates. Localization by in situ hybridization revealed heterogeneous expression in the epithelium beyond 6 weeks of age that was maintained through pregnancy, then became homogeneous during lactation. We also hypothesized that PRLR expression is co-associated with P receptor (PR) function. As for PRLR, the level of PR mRNA increased during puberty and declined during pregnancy, then by contrast, disappeared during lactation. In the mammary glands of virgin and pregnant mice the heterogeneous distribution of mRNA for PRLR and PR in the mammary epithelium co-localized. Consistent with our hypothesis, we identified that PRLR levels are increased in mice that lack cEBPβ and have increased PR expression, whereas mice lacking PR have reduced PRLR expression. These observations led us to test whether P and PRL interact during the stimulation of cell proliferation in ovariectomized female mice. Whereas P and PRL alone had no effect on cell growth, the combination of P+PRL induced extensive cell division in both epithelial and stromal cells that led to ductal branching of the mammary gland. By contrast, E alone stimulated cell proliferation but abrogated the effect of P+PRL. The interactive effect of P and PRL could be recapitulated in vitro, indicating direct effects of these hormones on the mammary gland. These results demonstrate that rather than acting alone, ovarian and pituitary hormones interact to stimulate cell proliferation and branching morphogenesis within the normal mammary gland. Such interactions likely contribute to normal human breast development as well as tumor initiation and progression.

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THE ROLE OF ERBB-2 IN MOUSE MAMMARY GLAND DEVELOPMENT

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The four members of the ErbB family of receptor tyrosine kinases are expressed in breast epithelium and are implicated in mammary gland development. Although the direct role for each receptor has not been determined due to a complex ErbB signaling network, there is evidence suggesting possible roles for the various ErbB receptors in growth and differentiation of the mammary gland. The ErbB-4 receptor activation is linked to terminal differentiation of alveolar structures in the mouse mammary gland. The ErbB-2/Neu/HER-2 oncogene is amplified or overexpressed in 30% of human breast cancers. The purpose of this research is to provide a direct analysis of the individual ErbB-2 and ErbB-4 receptors in the physiologically relevant context of the mouse mammary gland.

A direct analysis of the role of ErbB-2 in mouse mammary gland development has been deterred by the complexity of the ErbB signaling network and by the embryonic lethality of ErbB-2 null mice at E11. Genetic rescue of the lethal cardiac defect in ErbB-2 null mice results in embryos which die at birth due to severe peripheral nervous system defects. However, the rescued ErbB-2 null embryos' pre-natal mammary gland development is sufficient for in vivo transplantation techniques to be utilized for a post-natal ErbB-2 loss of function analysis. Mammary buds from rescued ErbB-2 null female embryos at E13.5-15.5 were transplanted into cleared fat pads of 3-week old female RAG1-/- mice. The transplanted glands were examined in virgin mice at 4 weeks, 7 weeks, and 13 weeks post-transplantation, and in pregnant mice. ErbB-2 null transplanted glands displayed epithelial growth with altered ductal structures compared to outgrowths from wild-type buds.

The role of ErbB-4 in the various stages of mouse mammary gland development will also be determined by a loss of function analysis with the use of genetically rescued ErbB-4 null mice. Elucidating the separate roles of the ErbB-2 and ErbB-4 receptors in normal mammary gland development may aid in understanding the roles of these receptors in breast cancer.

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FUNCTIONAL CHARACTERIZATION OF SNAIL AND SLUG ZINC-FINGER PROTEINS IN BREAST TISSUE

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Using the yeast one-hybrid screening approach with a human breast tissue hybrid cDNA library, Snail and Slug zinc-finger proteins were identified to interact with the CREaro element (cAMP-responsive element) in the promoter I.3 region of the human aromatase gene. These proteins have been shown as transcriptional repressors in many animal species including human, involved in mesoderm differentiation, epithelial-mesenchymal transition, brachial development and branching, neural crest cell migration, apoptosis and E-cadherin expression. Aromatase is an estrogen synthetase, and estrogen plays a critical role in breast cancer progression. This enzyme is expressed at higher levels in breast cancer tissues and cell lines than normal tissues, and its expression is driven by promoter I.3 in breast tumors whereas normal tissues generally use promoter I.4. Results from previous experiments on Snail and Slug proteins indicate that they act as repressors that down-regulate the expression of aromatase in normal breast tissue by suppressing the function of promoter I.3. In order to better understand the functions of these two zinc-finger proteins in human breast tissue, we stably transfected Snail, Slug and their antisense cDNAs in MCF7 (breast cancer cell line) and MCF10A (non-cancer breast epithelial cell line), respectively. These transfected cell lines were examined using three-dimensional culture in Matrigel. While MCF10A cells formed acinar structures containing a single layer of polarized growth-arrested cells in Matrigel, MCF10A cells transfected with the Snail antisense plasmid lost the ability to form the acini-like structure. MCF7 cells are Snail negative and Slug negative, and form disorganized structures in Matrigel. Interestingly, Snail or Slug over-expressing MCF7 cells form acini-like structure with lumen. These findings strongly suggest that Snail and Slug proteins are involved in cell polarization and acini formation. Immunohistochemistry is being carried out to investigate the localization of protein markers that are specific for cell polarization and lumen formation. This is the first report demonstrating that Snail and Slug zinc-finger proteins play a role in breast tissue differentiation and mammary duct development.

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THE HEDGEHOG SIGNALING INHIBITOR CYCLOPAMINE AFFECTS CANCER CELL GROWTH AND MAMMARY GLAND FUNCTION

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The hedgehog signal transduction network mediates inductive tissue interactions during the normal development of virtually every mammalian organ, including mammary gland. In skin, mutations in hedgehog network genes can increase hedgehog signaling inappropriately, leading to Basal Cell Carcinoma (BCC), the most common of all human cancers. Given that the mammary gland is a specialized skin derivative, we hypothesized that network mutations may function similarly in the development of breast cancer.

We have shown that loss-of-function mutations of at least two network genes, Patched-1 (Ptc1) and Gli2, lead to histological changes in mouse mammary glands similar to those observed in hyperplasias and ductal carcinoma in situ (DCIS) in women, supporting a mammary tumor suppressor role for these genes. If the mechanics of hedgehog signaling are conserved between skin and mammary gland, we envisioned that mutation of either the Ptc1 or Gli2 would result in improper activation of the signaling network via inappropriate activity either of Smo or of the hedgehog proteins themselves.

To test this hypothesis, we assayed the ability of a hedgehog signaling inhibitor, cyclopamine, to prevent or reverse Ptc1-induced dysphasia. We also tested the effect of cyclopamine on normal ductal development. In both cases, cyclopamine failed to have any demonstrable effect. However, when tested during the pregnancy to lactation transition, cyclopamine was able to inhibit lactation in a manner indistinguishable from Ptc1 overexpression in a transgenic mouse model. In a cell culture model, we demonstrate that cyclopamine can either slow the growth or kill MCF7 cells in vitro in a dose-dependent manner.

Coupled with concurrent phenotypic analysis of other network genes these observations suggest that Ptc1 and Gli2 functions are required for ductal development in the absence of hedgehog signaling, but that active hedgehog signaling is not. Our data suggest the need for a tightly regulated balance of network gene function, from which deviation in either direction can lead to DCIS-like hyperplasia and, in some cases, adenocarcinoma. Thus, hedgehog signaling inhibitors may only show effects on growth in a select class of human breast cancers.

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IDENTIFYING EFFECTORS OF PROLACTIN RECEPTOR SIGNALING DURING ALVEOLOGENESIS IN VIVO

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Prolactin signaling is essential for mammary gland development by promoting alveologenesis as well as alveolar differentiation. It has also been proposed that prolactin signaling may be involved in pathogenesis of human breast cancer. By binding to its receptor, prolactin signaling activates STAT transcription factors via JAK-2 kinase as well as activates MAP kinase pathway. However, few downstream effectors of these pathways are known, especially those responsible for the mitogenic responses to prolactin during alveologenesis. By genetically engineering a recombinant receptor with FKBP dimerization domain and a truncated prolactin receptor, we can regulate the chimerical receptor signaling in the cells via the administration of a chemical dimerizer, therefore mimicking wild type prolactin receptor signaling in the absence of prolactin or wild type prolactin receptor. Transgenic mice with the chimerical receptor have been generated and bred into a prolactin receptor knockout background where no endogenous prolactin signaling is present. We will present our experimental strategy and progress in using these reagents in identifying downstream effectors of prolactin signaling pathway in mice of late pregnancy stage. Information from both the acute and secondary responses to the receptor activation in vivo will help us in understanding the mechanisms of prolactin function in promoting mammary cell growth and differentiation.

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THE ROLE OF C-MYB IN BREAST DEVELOPMENT AND CANCER

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c-myb, along with A-myb and B-myb, belongs to the myb gene family which codes for nuclear proteins that bind DNA in a sequence-specific manner and function as regulators of transcription. There is a large body of evidence to suggest a role for c-myb in breast development and breast cancer, and it is known that c-myb is one of the most frequently altered genes in breast cancer. c-myb is highly expressed in all estrogen receptor positive (ER+) breast tumors as well as ER+ mammary carcinoma cell lines. In addition, our in situ hybridization studies show that c-myb is expressed at high levels in ductal cells of breast tissue from virgin and pregnant mice but is down-regulated in breast tissue of lactating mice. These observations suggest that c-Myb might play a critical role in estrogen-mediated ductal cell proliferation. To address the role of c-myb in mammary development and cancer, we propose to create c-myb conditional knockout mice where the expression of this gene is interrupted specifically in the mammary gland using embryonic stem (ES) cell technology and the Cre-lox system. We are in the process of generating chimeric mice by injecting our conditional c-myb deletion ES clonal cells into blastocysts of C57B/L6 pseudopregnant mice. Once we get chimeric mice, they will be bred to homozygosity for carrying conditional c-myb deletion alleles and, additionally, to bear either a MMTV-cre or a WAP-cre transgene. The generation of breast-specific c-myb conditional knockout mice will afford us the opportunity to dissect the role of c-myb in normal development as well as gain insight into the role of its aberrant expression in breast cancer. A detailed molecular understanding of how c-myb contributes to tumor progression is of major importance for future therapy.

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c-Src-SPECIFIC ACTIVATION OF THE ESTROGEN RECEPTOR IS REQUIRED FOR NORMAL MAMMARY GLAND DEVELOPMENT

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The proliferative and differentiation potential of the mammary gland is dependent on its ability to respond to both steroid and peptide growth factors. Although several studies suggest that crosstalk between various cytoplasmic factors and the steroid receptors occur, the precise molecular mechanism and functional consequences of these interactions remain to be elucidated. Here we demonstrate that a functional c-Src tyrosine kinase is required for the mammary epithelial cell to respond to estrogen. To assess the role of c-Src in mammary gland development, we performed wholemount analyses on mammary glands derived from mice lacking a functional c-Src protein. In contrast to wild type females, this analysis reveals that *c-src* null females display a dramatic defect in ductal outgrowth. Because ductal outgrowth of the mammary gland is dependent on the ability of the mammary epithelium to correctly respond to hormonal stimulation, we assessed whether the c-Src deficient epithelium could respond to exogenous estradiol stimulation. The result of this analysis reveals that mammary gland explants were incapable of efficiently phosphorylating the estrogen receptor, activating the MAP kinase pathway or inducing the expression of cyclin D1 in response to estrogen stimulation. Consistent with these observations, expression of a dominant negative inhibitor of c-Src (Src251) in MCF-7 cells results in a defect in the ability to activate MAP kinase in response to estrogenic stimulation. Furthermore, MCF-7 ERE3:LUC cells that express Src251 display an inability to efficiently induce reporter activity, suggesting that c-Src can directly effect ERE mediated transcription. Taken together, these observations suggest that c-Src is a critical mediator of estrogen action in the mammary epithelium.

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EXPRESSION OF CLAUDIN 7 IN THE MOUSE MAMMARY EPITHELIUM

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The claudins are a newly discovered family of proteins usually associated with tight junctions. Expression of one particular isoform of claudins, i.e., claudin 7, has been associated with breast tumors (Nacht M, Ferguson AT, Zhang W, et al. Cancer Res 1999;59:5464-5470). We have examined claudin-7 expression in mouse mammary tumors and during the development of the normal mammary gland using real time RT-PCR. We found that claudin 7 mRNA is increased more than 300 fold in early pregnancy, remains constant during lactation and is down-regulated at involution when compared to total RNA in the gland. However, the ratio of claudin 7 to an epithelial cell marker, cytokeratin-19 remains nearly constant through development suggesting that claudin-7 is a constitutive component of mammary epithelial cells. This hypothesis was confirmed by in situ localization of the mRNA to the mammary epithelium in mammary glands from virgin, pregnant and lactating mice. In contrast claudin 1 was most highly expressed in the virgin, claudin 3 was highly expressed during pregnancy and claudin 8 was significantly expressed only during lactation. Immunolocalization of claudin 7 in the normal mammary gland produced the surprising result that claudin-7 overlaps little with a marker of tight junctions, ZO-1, but rather appeared to be associated with both lateral and basal membranes of the cell. Claudin 1, on the other hand was localized to tight junctions in the epithelium of the virgin gland. These findings suggest that changes in the claudin complement play a significant role in the developmental changes of the mammary epithelium. In a panel of mouse mammary tumors the ratio of claudin-7 mRNA to cytokeratin mRNA was about 10 times higher than in the normal gland suggesting that the molecule might be used as a marker for breast cancer cells under certain circumstances.

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TRANSDUCTION OF THE MAMMARY EPITHELIUM WITH ADENOVIRAL VECTORS

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Up the teat microinjection of adenoviral vectors presents a non-invasive, non-inflammatory delivery system to study gene expression in the mammary epithelium. An E1/E3 deleted adenoviral vector (human adenovirus type 5) encoded with either LacZ or GFP reporter genes was injected into the fourth (LacZ) or third (GFP) mammary gland of mice at various stages of mammary gland development. 14C-sucrose was injected intraductally on the day of sacrifice to test the status of tight junctions and glands were excised to examine evidence of mastitis. Doses of 107 pfu (fourth mammary gland) or 2.6 x 106 pfu (third mammary gland) injected into day 17 pregnant mice showed minimal inflammation after 3 days. However, significant mastitis resulted after 7-10 days even with optimal doses of the adenovirus. Up to 40% of alveoli can be transduced at day 17 of pregnancy and up to 25% of the total epithelium can be transduced in the portion of the gland proximal to the teat. Although adenovirus can transduce the epithelium at any stage of mammary gland development, transduction gives the least inflammation in the late pregnant animal and can be maintained into early lactation. These findings demonstrate that up the teat microinjection of adenoviral vectors provides a versatile method of changing gene expression in cells of the mammary epithelium.

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GALANIN REGULATION OF MAMMARY LOBULOALVEOLAR DEVELOPMENT

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Galanin is a trophic neuropeptide involved in neuronal development and neuroendocrine regulation, and is also expressed by breast cancer cells indicating a possible role in mammopoiesis. Galanin and galanin receptors were found to be differentially expressed in the mammary gland during pregnancy. Null mutation of the galanin gene resulted in reduced lobuloalveolar development and lactational failure accompanied by decreased piuitary secretion of prolactin and an increase in relative phosphorylated prolactin levels. Treatment of wild-type mice with a molecular mimic of phosphoprolactin inhibited alveolar growth and caused lactational failure. Unmodified prolactin restored lactation in galanin knockout mice, however, rescue of lobuloalveloar development was incomplete. Transplantation of galanin knockout mammary epithelia to wild-type hosts demonstrated galanin does not have an essential autocrine/ paracrine role in mammary development. A direct endocrine role was demonstrated by culture of whole mammary gland explants, where addition of galanin resulted in an increase in size and a four-fold increase in the number of lobuloalveoli produced by lactogenic hormones alone. These data demonstrate that galanin is critical for mammary lobuloalveolar development and differentiation during pregnancy, acting directly on the mammary gland and also indirectly as a positive regulator of Prl secretion and phosphorylation during mammopoiesis.

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LEPTIN REGULATION OF MAMMARY CELL GROWTH

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The risk of developing breast cancer after menopause rises with obesity, although the cause is unknown. The expanding adipocyte (i.e., fat cell) population during obesity may contribute directly to breast cancer by providing excess factor(s) that maintain normal growth. An intriguing candidate is leptin, a protein produced almost exclusively by adipocytes. To test the hypothesis that leptin regulates mammary epithelial cell growth, a mouse mammary epithelial cell line, HC11, was incubated with increasing doses of leptin (0-100 ng/ml) either in the presence or absence of insulin (50 ng/ml) and epidermal growth factor (EGF; 10 ng/ml). Leptin alone had no effect on mammary epithelial cell growth, whereas leptin prevented cellular proliferation in the presence of insulin and EGF. Only the lowest concentration of leptin (1 ng/ml) reduced DNA synthesis. These results suggest that leptin may be a potential inhibitor of mammary epithelial cell proliferation when leptin concentrations are low, but not when concentrations are high as occurs with obesity. Although changes were detectable, they may have been limited by low leptin receptor expression due to the culture of cells on plastic. In an effort to increase receptor expression and maximize responses, HC11 cells were cultured in a collagen matrix, thereby representing a more natural environment. This three-dimensional system generated differential mRNA expression of both long and short receptor isoforms. With collagen alone, the long leptin receptor isoform was prevalent. In contrast, addition of insulin and EGF to the medium altered expression so that the short receptor isoform was more prevalent. The ability to induce differential expression of these receptors is critical as these receptors vary depending upon stage of mammary gland development. The results of these and subsequent studies will contribute directly to the knowledge of mammary gland development and possibly tumor development by providing new information regarding the signaling pathways between the adipocyte-rich stroma and mammary epithelial cells. Understanding these pathways in relation to both normal and pathologic mammary cell growth is imperative because of the greater risk of breast cancer that occurs with obesity.

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ISOLATION AND CHARACTERIZATION OF MAMMARY STEM CELLS

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Mammary epithelium can functionally regenerate upon transplantation. This renewal capacity has been classically ascribed to the function of a multipotent mammary gland stem cell population, which has been hypothesized to be a primary target in the etiology of breast cancer. Several complementary approaches were employed in this study to identify and enrich mammary epithelial cells that retain stem cell characteristics. Using long-term BrdU labeling, a population of label retaining cells (LRCs) that lack expression of differentiation markers has been identified. LRCs isolated from mammary primary cultures were enriched for stem cell antigen-1 (Sca-1) and Hoechst dye-effluxing "side population" properties. Sca-1pos cells in the mammary gland were localized to the luminal epithelia using Sca-1+/GFP mice, were progesterone receptor negative, and did not bind peanut lectin. Finally, the Sca-1pos population is enriched for functional stem/progenitor cells, as demonstrated by its increased regenerative potential compared to Sca-1neg cells when transplanted into the cleared mammary fat pads of host mice.

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HUMAN CHORIONIC GONADOTROPIN AND HOX GENES IN HUMAN BREAST EPITHELIAL CELLS

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The placental hormone human chorionic Gonadotropin (hCG) stimulates mammary gland differentiation and inhibits tumor progression. Because class I homeobox genes (Hox) are involved in mouse mammary gland development and in breast cancer, we hypothesized that hCG's effect is mediated by HOX gene activation. Reverse transcription-polymerase chain reaction (RT-PCR) was used for studying the expression of 39 known HOX genes in MCF-10F, MCF-7, and MDA-MB-231, an immortal and two cancer breast cell lines. Thirty-five of the 39 class I homeobox genes analyzed were expressed by the three cell lines; four, HOXA2, B1, C4, and C9 were not detected in any of them. Semi-quantitative RT-PCR technique was used for studying the expression patterns of these genes in cells treated with 5 μg of recombinant hCG (r-hCG)/ml for 1, 5, 10, 24, 48 and 96 hrs. In MCF-10F cells the 24-hr treatment up-regulated HOXD10 (3.8 folds), D11 (4.2 folds), and D13 (5.8 folds), whereas at 48 hrs of treatment only D8 was up regulated 4.2 folds. The silent gene HOXA2 was transiently detected at 1 hr of treatment. In MCF-7 cells, r-hCG treatments of 10 and 24 hrs resulted in up regulation of HOXB3 (2.5 folds), B8 (1.5 folds) and D8 (2.2 folds). In MDA-MB-231 cells, 1 and 5 hr-treatments up regulated HOXC8 (2.0 folds), C12 (2.0 folds), D8 (2.4 folds) and D11 (3.8 folds). Although HOXD8 was activated in the three cell lines, this effect varied with the time of treatment. The types of genes activated also varied in normal immortalized and in cancer cell lines, indicating that hCG plays a role in the regulation of HOX genes in human breast epithelial cells, but the effect is modulated by the biological characteristics of the cells.

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THE HYPOXIC RESPONSE IS CRITICAL FOR NORMAL MURINE MAMMARY GLAND DEVELOPMENT

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In response to hypoxia, or the exposure to low levels of oxygen, tissues try to restore homeostasis by regulating cellular metabolism and by inducing angiogenesis. The expression and protein stability of hypoxia-inducible transcription factor (HIF)-1alpha is increased rapidly under hypoxic conditions, inducing several genes implicated in the regulation of glycolysis, angiogenesis and apoptosis. In contrast, under normoxic conditions, the HIF-1alpha protein is degraded via targeted ubiquitination; this process is controlled by its interaction with the von Hippel-Lindau (VHL) tumor suppressor protein. Therefore, deletion of VHL leads to constitutive expression of HIF-1alpha protein, even at normoxia. Recently, HIF-1alpha protein has been demonstrated to be overexpressed in poor grade, highly proliferating breast tumors. Yet, it is not known if this overexpression promotes breast tumorigenesis or if the hypoxic response is essential for normal mammary gland development. To address these questions, we have used a conditional cre/*lox* strategy to delete either HIF-1alpha or VHL upon activation of either the MMTV-Cre or WAP-Cre transgenes. Mice that lack HIF-1alpha develop fewer and less well-differentiated alveoli during their first pregnancy. At lactation, these mice fail to properly nourish their litters, resulting in reduced pup growth, and eventually, pup death. Milk collected from the HIF-alpha-deficient mice contained 40% less lactose, the primary carbohydrate component of milk, than milk collected from control mice, possibly explaining the growth phenotype. Transplantation experiments suggest that these phenotypes are epithelial cell autonomous. Upon multiple rounds of breeding, loss of VHL impairs pup growth, similar to loss of HIF-1alpha. In contrast to the phenotype observed in the HIF-1alpha crosses, glands lacking VHL are hypervascularized and contain disorganized alveoli that appear to be surrounded by a large number of red blood cells, implying that deletion of VHL may lead to mammary hyperplasia and/or loss of proper extracellular matrix interactions. Based on these results, we propose that either deletion or overexpression of HIF-1alpha (via loss of VHL) impairs the developmentally-regulated metabolic and angiogenic pathways necessary for lactation.

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REGULATION OF MAMMARY EPITHELIAL CELL GROWTH BY CCAAT/ENHANCER BINDING PROTEIN (C/EBP) BETA

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Gene knockout studies show that the transcription factor, C/EBPbeta, is critical for growth and differentiation of the mammary gland during puberty, pregnancy, lactation, and involution. Three forms of C/EBPbeta can be expressed in cells by alternative translation initiation. C/EBPbeta-1 and C/EBPbeta-2 activate transcription; C/EBPbeta-3 is a truncated form that represses transcription. We have observed a striking difference in the expression profiles of C/EBPbeta-1 and –2 in normal and neoplastic human mammary epithelial cells (MECs) derived from both primary tissues and established cell lines. Specifically, C/EBPbeta-1 is expressed in normal human mammary epithelial cells (HMECs) from reduction mammoplasties and ductal secretory epithelial cells harvested from human breast milk, but is absent in all breast cancer cell lines examined, which express only the C/EBPbeta-2 transactivator. Normal breast tissue from reduction mammoplasties expressed exclusively C/EBPbeta-1, whereas 7 of 10 surgical primary breast tumor samples acquired elevated C/EBPbeta-2 levels.

To further study whether the two C/EBPbeta activator isoforms carry out distinct roles in MECs, we have exploited recombinant retroviral technology to selectively overexpress either epitope-tagged C/EBPbeta-2 or C/EBPbeta-1 in various human mammary cell lines. C/EBPbeta retroviruses with or without a marker, green fluorescent protein, were generated using a hybrid Epstein-Barr virus (EBV)/retroviral vector construct (LZRSpBMN-Z) developed and provided to us by G. Nolan (Stanford University). We found that elevated levels of tagged C/EBPbeta-2 resulted in dramatic transformation of a normal human mammary epithelial cell line, MCFA. MCF10A cells engineered to overexpress C/EBPβ-2 form foci, gain anchorage independence, express markers associated with having undergone an epithelial to mesenchymal transition (EMT), and acquire an invasive phenotype. Interestingly, when C/EBPβ-1 was overexpressed in the highly invasive MDA 231 cells, the invasive behavior in culture of these cells was completely reversed. C/EBPβ-1 is not expressed in MDA 231 cells; reintroducing this isoform led to the adoption of a fused/spherical morphology in Matrigel, which is typical of less invasive breast cancer cell lines. Based upon these observations, and other data, we propose that C/EBPβ-2 activates genes which promote invasive cell growth, whereas the biological role of C/EBPβ-1 is to activate differentiation specific gene transcription. These studies provide supportive evidence that deregulated expression of C/EBPβ-2 contributes to malignant conversion of the human breast.

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AN ADJUNCT MAMMARY EPITHELIAL CELL POPULATION IN PAROUS FEMALES: ITS ROLE IN FUNCTIONAL ADAPTATION, TISSUE RENEWAL, AND NEOPLASTIC TRANSFORMATION

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Mammary gland biologists have long assumed that differentiated secretory epithelial cells undergo programmed cell death at the end of lactation and that the alveolar compartment is reconstituted from undifferentiated precursor cells in subsequent pregnancies. It is generally agreed that the remodeled gland in a parous animal resembles that of a mature virgin on the morphological level. However, a number of physiological differences have been noted in comparing the responses of mammary epithelia from nulliparous versus parous females to hormonal stimulation and carcinogenic agents. Here, we present genetic evidence that an involuted mammary gland is fundamentally different from a virgin gland despite its close resemblance in the morphology. This difference results from the formation of a new mammary epithelial cell population that originates from differentiating cells during pregnancy. In contrast to the majority of fully committed alveolar cells, this epithelial population does not undergo cell death during involution and remodeling following a lactation period. We show that these cells can function as alveolar progenitors in subsequent pregnancies, and they can play an important role in functional adaptation in genetically engineered mice, which exhibit a reversion of a lactation-deficient phenotype in multiparous animals. In transplantation studies, this parity-induced epithelial population shows the capacity for self-renewal and contributes significantly to the reconstitution of the resulting mammary outgrowth (i.e., ductal morphogenesis and lobulogenesis). We propose that this parity-induced population contributes importantly to the biological differences between the mammary glands of parous and nulliparous females.

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PSEUDOPHOSPHORYLATED PROLACTIN (S179D PRL) INHIBITS GROWTH AND PROMOTES DIFFERENTIATION IN THE RAT MAMMARY GLAND

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Prolactin (PRL) has long been recognized as a hormone having both proliferative and differentiative activities in the mammary gland. Current theory proposes that it is the co-existing steroidal environment which dictates whether PRL is mainly proliferative, as it is during pregnancy, or mainly differentiative, as it is during lactation. Our hypothesis, on the other hand, suggests that the form of PRL is also important in that it dictates a primarily proliferative versus primarily differentiative response, with unmodified PRL (U-PRL) producing the former and phosphoPRL, the latter. To test this hypothesis, recombinant versions of each PRL were administered to rat dams throughout pregnancy at a rate resulting in circulating concentrations of 50 ng/ml. Measurement of progesterone, estradiol and corticosterone showed no effect of the administered PRLs on these other mammotropic hormones. Histological and morphometric analysis showed U-PRL to cause an increase in gland size (102 ± 7 mm^3, U-PRL v 67 ± 5 mm^3, control, $p < 0.01$), while the molecular mimic of phosphoPRL, S179D PRL, decreased gland size (40.2 ± 4.6 mm^3, $p < 0.05$). The number of pup implantation sites was indistinguishable among groups (13 ± 1), as was the level of expression of placental lactogen II mRNA in placentae from day 19.5 of gestation (Northern blot). In addition to an overall decrease in size of the S179D PRL-treated mammary gland, there was a decrease in area occupied by alveoli to almost half the control level ($p < 0.001$). This resulted in lactational failure. In contrast, Northern analysis of β-casein expression normalized to 18s RNA demonstrated decreased expression in mammary glands from the U-PRL-treated dams and increased expression in the S179D PRL-treated dams ($p < 0.05$). *In vitro* studies with the rodent mammary cell line, HC11, confirmed the superior ability of S179D PRL to promote β-casein gene expression (7 fold more potent than U-PRL, $p < 0.01$). Further experiments with non-pregnant animals confirmed that effects on the mammary gland were not secondary to changes in placental lactogens and that pregnancy levels of progesterone were not required. We conclude that U-PRL is primarily proliferative and that S179D PRL is primarily differentiative in the pregnant mammary gland, although there is some degree of cross activation. Ongoing experiments include 1) analysis of the interplay among these two PRLs and the other mammotropic hormones using mammary tissue explants, and 2) testing of the hypothesis that exposure to additional phosphorylated PRL during pregnancy would enhance pregnancy-related refractoriness to carcinogenesis.

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MOLECULAR EMBRYOLOGY OF THE NONHUMAN PRIMATE

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By definition, every species has features unique to its own biology. In our lab, we are ultimately seeking to understand some of the factors that control these differences. In particular, we are examining the differences between mouse and primate early development.

Primate and mouse display marked morphological differences upon their entrance to gastrulation. The most overt of these differences is the topological dissimilarity of the gross embryo. While the primate embryo develops as a planar epiblast within a spheroidal cytotrophoblastic shell, the mouse epiblast develops in a cupped shape, surrounded principally by a cylindrical shell of endoderm. At this stage, the bulk of the murine trophoblast is limited to the mesometrial pole of the cylinder, rather than encapsulated by the cytotrophoblast as in the primate embryo. Additionally, the appearance of the primate mesoderm is precocious with respect to that of the mouse. Primate extraembryonic mesoderm is evident in sectioned tissues prior to the establishment of a primitive streak or node. In the mouse, such mesoderm appears only after establishment of these structures.

To begin to understand the molecular reasons for these differences, we are developing a new model of non-human primate biology using interspecific chimeras produced within tetraploid mouse blastocysts. By using tetraploid blastocysts in combination with non-human primate embryonic stem cells, we are segregating the extraembryonic membranes of one species from the developing embryonic structures of the second species. The purpose of this is to provide a non-human primate model in which we can perform molecular tests that are currently only tractable in murine systems. Eventually, we would like to use this model to study differential gene regulation by using the power of embryonic stem cell technologies to perform gene-swap experiments between the developing mouse and our model of the developing primate. Additionally, we are extending previous morphological descriptions to a molecular comparison of specific markers that are expressed in key landmarks of early staged mouse embryos. By making a molecular comparison between non-human primate and mouse embryos, we will shed light on the underpinning nature of the morphological differences between the two species.

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PARITY-INDUCED CHANGES IN MAMMARY GENE EXPRESSION

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Epidemiologic studies have repeatedly demonstrated that women who undergo an early first full-term pregnancy have a significantly reduced lifetime risk of breast cancer. Similarly, rodents that have previously undergone a full-term pregnancy are highly resistant to carcinogen-induced breast cancer compared to age-matched nulliparous controls. The realization that specific reproductive endocrine events alter breast cancer risk in a predictable fashion raises the possibility that naturally occurring events known to decrease breast cancer risk might be mimicked pharmacologically. The desire to pursue this objective is heightened by the fact that while it is now possible to identify women who are at elevated risk for developing breast cancer, few interventions currently exist. As such, reducing breast cancer risk via hormonal manipulations designed to mimic naturally occurring endocrine events could represent an attractive alternative. It is to this end that an early first full-term pregnancy has been proposed as a logical paradigm on which to model the hormonal chemoprevention of breast cancer. The achievement of this goal, however, has been hampered by our lack of understanding of the mechanisms by which reproductive events alter breast cancer risk. Understanding these mechanisms will ultimately facilitate the design of safe and effective hormonal chemoprevention regimens as will the identification and use of intermediate molecular endpoints that accurately detect changes in the breast associated with changes in breast cancer risk.

We have used DNA microarrays to identify a panel of differentially expressed genes that reproducibly distinguishes, in a blinded manner, between the nulliparous and parous states of the mammary gland in multiple strains of mice and rats. We find that parity results in the persistent down-regulation of multiple growth promoting pathways, as well as the persistent upregulation of growth inhibitory pathways. Our studies further indicate that parity results in a persistent increase in the differentiated state of the mammary gland as well as lifelong changes in the hematopoietic cell types resident within the gland. These findings define a developmental state of the mammary gland that is refractory to carcinogenesis.

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